PB82-249319

Species Susceptibility to Delayed Toxic Neuropathy in Relation to In vivo Inhibition of Inhibition of Neurotoxic Esterase by Neurotoxic Organophosphorus Esters

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

30 Jun 81

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1. REPORT NO. EPA-600/J-81-238	2. JOURNAL ARTICLE
3. RE	PB82-249319
4. TITLE AND SUBTITLE: Species Susceptibility to Delayed Toxic Neuropathy in Relation to in vivo Inhibition of Neurotoxic Esterase by Neurotoxic Organophosphorus Esters	5. REPORT DATE
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): S.A. Soliman, R. Linder, J. Farmer, A. Curley	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: U.S. Envrionmental Protection Agency, Toxic Effects Branch, Environmental Toxicology Division, Research Triangle Park, North Carolina 27711	10. PROGRAM ELEMENT NO. ACSL1A
	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS: Office of Research and Development, Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES: Published in: J. of Tox.and Environ. Health, 9:189-197, 1982

16. ABSTRACT

Tri-o-cresyl phosphate (TOCP) and O-ethyl O-(4-cyanophenyl) phenylphosphonothioate (cyanofenphos, Surecide) were found to be delayed neurotoxicants. They were administered to chickens by gavage at 100 and 30 mg/kg·d for 15 d, respectively. In CD-1 mice neither TOCP nor cyanophenphos induced any of the usually recognized clinical symptoms of neuropathy when administered daily by gavage at 262 or 31.25 mg/kg·d for 30 d, respectively. In the chickens, TOCP and cyanofenphos produced about 98 and 90% in vivo inhibition of brain neurotoxic esterase (NTE) activity. In the mice, 24 h after the last daily dose, TOCP and cyanofenphos produced only about 50 and 40% in vivo inhibition of the brain NTE activity. Parathion [O,O-diethyl O-(4-nitrophenyl) phosphorothioate], at 2 or 6.75 mg/kg·d for 15 or 30 d, did not induce neuropathy in either chicken or mice and produced no significant in vivo inhibition of brain NTE activity at the end of the dosing regimen. The specific activity of NTE in control chicken brain crude homogenate was much higher than that in mouse brain homogenate. These results suggest that the differences between chickens and mice in susceptibility to neurotoxic organophosphates may be attributed to (1) inhibitor specificity of NTE forms in the brain in these two different animal species and/or (2) inability of the active metabolites of these neurotoxic compounds to reach the site of action.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

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SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT: Release to Public	19. SECURITY CLASS (This Report): Unclassified	21. NO. OF PAGES
	20. SECURITY CLASS (This page): Unclassified	22. PRICE

EPA-600/J-81-238
JOURNAL ARTICLE

SPECIES SUSCEPTIBILITY TO DELAYED TOXIC NEUROPATHY IN RELATION TO *IN VIVO* INHIBITION OF NEUROTOXIC ESTERASE BY NEUROTOXIC ORGANOPHOSPHORUS ESTERS

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Tri-o-cresyl phosphate (TOCP) and O-ethyl O-(4-cyanophenyl) phenylphosphonothioate (cyanofenphos, Surecide) were found to be delayed neurotoxicants. They were administered to chickens by gavage at 100 and 30 mg/kg•d for 15 d, respectively. In CD-1 mice neither TOCP nor cyanophenphos induced any of the usually recognized clinical symptoms of neuropathy when administered daily by gavage at 262 or 31.25 mg/kg•d for 30 d, respectively. In the chickens, TOCP and cyanofenphos produced about 98 and 90% in vivo *inhibition of brain neurotoxic esterase (NTE) activity. In the mice, 24 h after the last daily dose, TOCP and cyanofenphos produced only about 50 and 40%* in vivo *inhibition of the brain NTE activity. Parathion [O,O-diethyl O-(4-nitrophenyl) phosphorothioate], at 2 or 6.75 mg/kg•d for 15 or 30 d, did not induce neuropathy in either chicken or mice and produced no significant* in vivo *inhibition of brain NTE activity at the end of the dosing regimen. The specific activity of NTE in control chicken brain crude homogenate was much higher than that in mouse brain homogenate. These results suggest that the differences between chickens and mice in susceptibility to neurotoxic organophosphates may be attributed to (1) inhibitor specificity of NTE forms in the brain in these two different animal species and/or (2) inability of the active metabolites of these neurotoxic compounds to reach the site of action.*

INTRODUCTION

Toxic distal axonopathy (Schaumburg and Spencer, 1979), or in general delayed toxic neuropathy, is characterized pathologically by distal axonal degeneration and secondary demyelination of the central and peripheral

We are grateful for the technical assistance of Ms. Rosanne Novak and Ms. Faye Gregory.

This work was supported by Environmental Protection Agency contract 68-02-3252.

This paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Journal of Toxicology and Environmental Health, 9:189-197, 1982
Hemisphere Publishing Corporation
0098-4108/82/020189-09$2.25

nervous axons and by a time delay of 8–14 d before onset of clinical pathological symptoms after single or multiple doses (Cavanagh, 1964, 1973). Many species of vertebrates, including humans, monkey, dogs, tigers, cats, rabbits, cows, pigs, sheep, mice, rats, and hens, are more or less susceptible to delayed neuropathy (Eto, 1974). Cats and adult hens are highly sensitive to the delayed neurotoxicity of organophosphorus compounds (Barnes and Denz, 1953; Cavanagh, 1954; Taylor, 1967). Rabbits and rats require repeated doses of these compounds for the same axonal degeneration (Barnes and Denz, 1953). Repeated doses of mipafox (*N,N'*-diisopropylphosphorodiamidic fluoride), for example, induce in rats within 3–4 mo a typical peripheral neuropathy (Majno and Karnovski, 1961). Sheep and pigs are susceptible to delayed neuropathy caused by organophosphorus compounds (Dobson, 1977; Malone, 1964; El-Sebae et al., 1977, 1979). Humans are susceptible to the neurotoxic organophosphates (Smith et al., 1930; Bidstrup et al., 1953; Smith and Spalding, 1959). Previous experimental studies did not indicate whether the differences in species susceptibility arise from special properties of the nerve cells of different animals or depend on other factors.

It was concluded that there is a significant relation between the delayed toxic neuropathy of organophosphorus compounds and their reaction with a brain protein in hens (Johnson, 1968, 1969, 1975; Aldridge and Johnson, 1971). The reaction was reported to be phosphorylation of the active site of an esterase, referred to as neurotoxic esterase (NTE), which hydrolyzes phenyl phenylacetate (PPA) or phenyl valerate (PV) and can be distinguished analytically from other PPA- or PV-hydrolyzing esterases by its response to specific inhibitors (Johnson, 1975). After more than 12 yr of studies with this enzyme, it was suggested that a neurotoxic organophosphorus compound must be a potent inhibitor of NTE activity *in vivo* to cause delayed neurotoxic effects (Johnson, 1968, 1975; Johnson and Lotti, 1980). In a recent study of the subcellular distribution of NTE activity in the brains of different animals, the specific activity of NTE in lamb brain was found to be much higher than that in mouse brain (Soliman et al., 1980). This might help explain the characteristic species variations in susceptibility to NTE inhibitors, which are known to be potent delayed neurotoxic agents (Soliman et al., 1980).

The purpose of this study was to investigate the neurotoxicity of some organophosphorus compounds in chickens (as susceptible species) and in mice (as less susceptible species) with special reference to the *in vivo* inhibition of NTE activity.

MATERIALS AND METHODS

Chemicals

Parathion [*O,O*-diethyl *O*-(4-nitrophenyl) phosphorothioate] and its oxon analog paraoxon were obtained from Chemical Service, Inc.,

West Chester, Pa. TOCP (tri-*o*-cresyl phosphate) was obtained from Eastman Kodak Co. (Rochester, N.Y.). Technical 93% cyanofenphos [Surecide; *O*-ethyl *O*-(4-cyanophenyl) phenylphosphonothioate] was provided by Sumitomo Chemical Co. of Osaka, Japan. Mipafox and PV were synthesized (Soliman et al., 1980). All other chemicals used were of the highest purity available from Sigma Chemical Co. (St. Louis, Mo.) and Aldrich Chemical Co. (Milwaukee, Wis.).

Treatment and Care of Chickens

Adult white leghorn hens (1.5–2.0 kg) were obtained from Featherdown Farm (Apex, N.C.). Hens were divided into 5 groups of 12 birds each and allowed to adjust to the colony room conditions at unit 6 of the research farm, Department of Poultry Sciences, North Carolina State University (NCSU), for 2 wk before treatment. They were supplied with food and water *ad libitum*. The first group of chickens was kept as an untreated control group. The second group was treated orally with corn oil daily at 1 ml/kg·d for 15 d and kept as the vehicle control group. The third and fourth groups were given daily oral doses of 2 mg/kg parathion or 100 mg/kg TOCP for 15 d and kept as negative and positive controls, respectively. The fifth group received daily oral doses of 30 mg/kg cyanofenphos for the same period. Test concentrations were prepared by dissolving or suspending the compound in corn oil to prepare solutions equivalent to the test doses when administered in 1 ml/kg. In 15 d, chickens received cumulative doses approximately equivalent to the acute LD50 of TOCP and cyanofenphos for hens (Smith and Lillie, 1931; El-Sebae et al., 1980).

Treatment and Care of Mice

Male CD-1 mice 60 d old (30–35 g) were obtained from Charles River Breeding Laboratory (Wilmington, Mass.). Mice were divided into 5 groups of 10 mice each and allowed to adjust to the laboratory conditions for 3 wk. The first group of mice was kept as an untreated control group. The second group received daily oral doses of 10 ml/kg corn oil for 30 continuous d and was used as the vehicle control group. The third, fourth, and fifth groups were given daily oral doses of parathion, TOCP, or cyanofenphos at 6.75, 262.00, or 31.25 mg/kg respectively. Each daily dose was calculated to be equivalent to one-fourth of the LD50 determined for the compounds with mice of the same stock (unpublished data), so that mice were given almost 7.5 times the LD50 values of these compounds during the experiment.

Clinical Observations and Tissue Preparations

Hens and mice were observed daily before treatment for any abnormalities. Three chickens from each group were sacrificed by decapitation at 1, 7, 15, and 30 d after the first dose and 3 mice of each group were sacrificed at 1, 15, and 30 d after the first dose for assay of brain NTE

activity. Brains were taken from the chickens and mice at necropsy as quickly as possible and homogenized individually, using a Polytron homogenizer for 20 s and 30 or 3 ml Tris-HCl buffer for chicken and mouse brains, respectively. The Tris-HCl buffer was 50 m*M*, pH 8, and contained 0.2 m*M* EDTA. The homogenates were used directly to assay NTE activity. The remaining animals were observed during the 30-d experimental period for delayed neuropathy, manifested clinically by leg weakness, ataxia, and paralysis, and the effect was graded on an 8-point scale according to Cavanagh et al. (1961).

Assay of Neurotoxic Esterase Activity

The NTE activity in chicken and mouse brain homogenates was determined by the differential technique (Johnson, 1977) with slight modifications. Paired samples of the homogenate, each of 200 μl, were preincubated in Tris-HCl buffer (50 m*M*, pH 8) containing 2 m*M* EDTA for 20 min with paraoxon (40 μ*M*) plus either buffer (tube a) or mipafox (50 μ*M*) (tube b) in a final volume of 3 ml at 37°C. After preincubation, 20 μl dimethylformamidic solution of the substrate PV (25 mg/ml) was added and the incubation was continued for 10 min. The enzymatic reaction was stopped at the end of the incubation period by adding 2 ml sodium dodecyl sulfate (SDS) solution (1% w/v) in the same buffer but containing 4-aminoantipyrine (0.025% w/v). Tube contents were mixed immediately after addition of SDS, 1 ml aqueous potassium ferricyanide solution (0.4% w/v) was then added, and the resultant stable red color was recorded at 510 nm after 10 min against blanks from which PV was omitted. The nonenzymatic hydrolysis of PV under the same conditions was determined. NTE activity was calculated by subtraction of the remaining PV-hydrolyzing activity in tube b from that in tube a. The protein content of the homogenates was determined by the method of Lowry et al (1951).

RESULTS AND DISCUSSION

Clinical Observations

Acute effects. None of the treated mice showed signs of acute cholinergic poisoning during this experiment. Most of the parathion-treated chickens showed some or most of the typical symptoms of moderate cholinergic poisoning 2–4 h after the daily treatment, but they were found to be fully recovered by the next morning. None of the TOCP- or cyanofenphos-treated chickens showed any signs of acute toxicity during the first week of dosing.

Delayed neuropathy. None of the treated mice showed any signs of leg weakness or ataxia throughout the experiment. All the chickens in the untreated, vehicle control, and parathion-treated groups were normal and looked healthy during the last 15 d of the experiment.

TABLE 1. Clinical Assessment of Delayed Neuropathy Induced in Chicken by TOCP and Cyanofenphos

Treatment dose and period	Degree of ataxia[a] at different days after the first dose					
	Hen	10	15	20	25	30
TOCP, 100 mg/kg·d for 15 d	43	0	7	[b]		
	44	2	5	[b]		
	45	0	6	[b]		
	46	0	7	7	7	8
	47	0	6	8	7	8
	48	0	6	6	8	8
Cyanofenphos, 30 mg/kg ·d for 15 d	55	0	2	[b]		
	56	0	0	[b]		
	57	0	3	[b]		
	58	0	0	4	5	5
	59	0	2	6	6	7
	60	0	2	4	4	6

[a]Degree of ataxia: mild (1–2 points), slight (3–4 points), severe (5–6 points), and paralysis (7–8 points); for more detail see Cavanagh et al. (1961).
[b]Bird sacrificed at d 15.

Table 1 summarizes the delayed neurotoxic symptoms induced in the remaining chickens of the TOCP- and cyanofenphos-treated groups. The time of onset of ataxia in TOCP-treated chickens was earlier than in cyanofenphos-treated chickens. The degrees of ataxia in the TOCP group were more severe than in the cyanofenphos-treated group of chickens.

Clinical observations indicated that mice have low susceptibility to the neurotoxic organophosphates in comparison with chickens. In previous studies, mice developed neurotoxic symptoms following long-term treatment with leptophos [*O*-methyl *O*-(4-bromo-2,5-dichlorophenyl) phenylphosphonothioate] (El-Sebae et al., 1977). This may be attributed to (1) strain and environmental differences and/or (2) potency of the compounds tested. The minimum ataxic single doses of cyanofenphos in chickens are about 100–125 and 200–250 mg/kg when the treated birds are kept at about 35 and 7°C, respectively (unpublished data). Moreover, methyl-substituted phenylphosphonothioates were reported to be potent delayed neurotoxic agents in comparison with their ethyl analogs (Hollingshaus et al., 1979).

In vivo *inhibition of NTE activity.* The brain NTE activities of the corn oil-treated groups of chickens and mice were not significantly different from those of their corresponding untreated controls; therefore the mean activities in the two groups were calculated and used to determine the effect of other treatments. The specific NTE activities in the control chicken and mouse brain crude homogenates were found to be

TABLE 2. *In Vivo* Effect of Tested Organophosphorus Compounds on NTE Activity in Chicken and Mouse Brain

Treatment	Species	Daily dose (mg/kg)	Maximum no. of doses	Total maximum cumulative dose (mg/kg)	Percent of activity remaining after treatment (days from first dose)[a]			
					1	7	15	30
Parathion	Chicken	2.00	15	30.0	92.16 ± 6.44	105.12 ± 7.18	91.38 ± 8.15	109 ± 11.20
	Mouse	6.75	30	202.5	98.56 ± 7.18	–	108.15 ± 9.24	96.14 ± 8.78
TOCP	Chicken	100.00	15	1500.0	58.34 ± 2.48	19.67 ± 2.46	2.66 ± 3.12	50.22 ± 6.32
	Mouse	262.00	30	7860.0	90.18 ± 9.97	–	74.18 ± 6.22	50.19 ± 3.71
Cyanofenphos	Chicken	30.00	15	450.0	60.13 ± 1.98	23.92 ± 3.23	10.16 ± 2.18	68.56 ± 6.21
	Mouse	31.25	30	937.5	96.27 ± 5.18	–	81.08 ± 3.19	59.59 ± 5.22

[a]Mean ± SD.

(mean ± SD) 10.4 ± 0.7 and 3.1 ± 0.3 nmol phenol produced per milligram of protein per minute, respectively. These results indicate that the activity in chicken brain was much higher than that in mouse brain. This finding agrees with previous data on sheep and mouse brain NTE activity (Soliman et al., 1980). However, in future studies enzyme preparations of much higher purity will be used to clarify these differences. Table 2 shows the *in vivo* effects of daily doses of parathion, TOCP, and cyanofenphos on chicken and mouse brain NTE activity. The data indicate that parathion did not have a significant effect on NTE activity even though the chickens and mice received cumulative doses equivalent to 6 and 7.5 times the corresponding LD50 values, respectively, with no distinct signs or symptoms of neuropathy. TOCP or cyanofenphos after 15 continuous daily doses (total equivalent to the corresponding LD50) produced about 97 and 90% total inhibition of chicken brain NTE activity and distinct signs and symptoms of neuropathy. However, up to 7860 or 937.5 mg/kg TOCP or cyanofenphos administered in 30 daily doses did not induce signs of delayed neuropathy in mice and produced only about 50 or 40% *in vivo* total inhibition of brain NTE activity, respectively (Tables 1 and 2). These results are in agreement with the suggestion that genesis of the neuropathy requires a high degree of NTE inhibition (>70%) during or shortly after the dosing regimen (Johnson and Lotti, 1980).

The reelevation of chicken brain NTE activity following termination of the dosing regimen, as shown in Table 2, is assumed to be due to resynthesis of NTE rather than reactivation of inhibited NTE. This assumption is based on the high stability of aged NTE, which formed after phosphorylation of the enzyme by active metabolites of TOCP or organophosphonates (Johnson, 1980).

The data presented in Table 2 suggest that the differences between chickens and mice in susceptibility to the neurotoxic organophosphates may be attributed to (1) inhibitor specificity of NTE forms in brains of these two different species and/or (2) inability of the active metabolites of these neurotoxic compounds to reach the site of action in sufficient concentration. Differences in biotransformation, distribution and excretion processes, or bioavailability may be important for explaining the observations in mice.

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Received May 29, 1981
Accepted June 30, 1981